NEFA Elevation during a Hyperglycaemic Clamp Enhances Insulin Secretion

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Elevated non-esterified fatty acid (NEFA) levels may influence insulin secretion and contribute to the development of Type 2 DM. We investigated the effects of acute NEFA elevation in controls ($n = 6$) and subjects predisposed to Type 2 DM ($n = 6$) on basal insulin levels, and following glucose and arginine stimulation. Each subject had one study with a triglyceride (TG) plus heparin infusion (elevated NEFA levels) and another with normal saline. Twenty minutes after the TG or saline infusion began a glucose bolus was given and 10 min later a 90-min hyperglycaemic clamp (~ 9 mmol l^{-1}) was started. Intravenous arginine was given at 110 min. Elevated NEFA levels (~ 4000 μmol l^{-1}) did not enhance basal or first phase glucose stimulated insulin levels. During hyperglycaemia, NEFA elevation further increased insulin levels in both groups by 20–44 % ($p < 0.05$) and C-peptide levels by 17–25 % ($p < 0.05$). The post-arginine insulin levels during hyperglycaemia were increased by 45 % in the Type 2 DM-risk group ($p < 0.02$). The glucose infusion rate maintaining matched hyperglycaemia was similar during NEFA elevation and for saline control for both groups. We conclude that acute elevation of NEFA levels enhances glucose and non-glucose-induced insulin secretion. © **1998 John Wiley & Sons, Ltd.**

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Introduction

Elevated circulating levels of non-esterified fatty acids (NEFA) have been implicated in the pathogenesis of Type 2 diabetes mellitus (DM).[1,2] Acute elevation of plasma NEFA with a triglyceride (TG) infusion can produce insulin resistance.[3–5] However, frank hyperglycaemia and Type 2 DM imply defective insulin secretion as well as impaired action. Several studies have examined the effects of elevated plasma NEFA levels on insulin secretion in man, but with variable results.[6–12] Studies that elevated NEFA levels by an oral fat load[6,8,11,12] are complicated by the release of insulinotropic hormones that augment glucose-induced insulin secretion.[8,13] Use of an intravenous infusion of TG (with or without heparin) has produced discrepant data on the effect of elevated NEFA on basal insulin levels.[7,9,10,14] One of these studies demonstrated increased C-peptide but not insulin levels. More recently, it was shown that the acute insulin response to an intravenous glucose challenge during continuous NEFA elevation was increased at 6 h but decreased at 24 h,[15] while another recent study[16] demonstrated that a continuous dextrose infusion together with intravenous TG and heparin increased insulin secretory rates over 48 h.

In Type 2 DM the insulin response to non-glucose secretagogues such as arginine is preserved although the insulin secretory response is impaired when the prevailing level of glycaemia is taken into account.[17] Plasma NEFA levels are often elevated in Type 2 DM.[18,19] It may be that background elevation of NEFA levels contributes to the preserved response to non-glucose secretagogues in Type 2 DM. We therefore examined the effects of an acute elevation of circulating NEFA on basal, glucose-stimulated, and glucose-plus-arginine-stimulated insulin secretion. We have examined responses in subjects at high and low risk of Type 2 DM.

Subjects and Methods

Six non-obese subjects without a family history of diabetes were recruited to represent the most normal end of the spectrum of carbohydrate and lipid metabolism. Six older subjects with a high risk of developing Type 2 DM (4 with previous gestational diabetes and 2 with a strong family history of Type 2 DM in at least 1 first degree relative: 'Type 2 DM-risk') represented a group towards the abnormal 'prediabetic' end of the spectrum of carbohydrate and lipid metabolism. All had normal fasting plasma glucose levels of < 6.1 mmol l^{-1}. Recruit-

Abbreviations: GIR glucose infusion rate, HGP hepatic glucose production, NEFA non-esterified fatty acids, OHB 3 hydroxybutyrate, TG triglyceride

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ment was via advertisements in University campus magazines and through medical records of the Royal Hospital for Women, Sydney. Subjects with any significant medical illnesses or who took any medications were excluded. All the studies were approved by the St Vincent's Hospital Research Ethics Committee and each subject gave informed consent.

After an overnight fast, an 18 gauge intravenous cannula was inserted retrogradely into a wrist vein of a hand heated with a thermostatically controlled pad for collection of 'arterialized' blood samples. In the contralateral arm, a 30 cm 16 gauge long-line was inserted for the infusion of 50 % (w/v) glucose, and Intralipid® (Travenol, Sydney, Australia) plus heparin or normal saline; 20 % Intralipid is a fat emulsion containing 20 % TG, 1.2 % phospholipids, and 2.25 % glycerol. Each subject had two studies performed on separate days in random order. Both studies consisted of a hyperglycaemic clamp (~ 9 mmol l^{-1}) combined with a 20 % Intralipid® plus heparin infusion on one day, and a normal saline infusion on the other day. The Intralipid or normal saline was infused at a rate of 48 ml h^{-1} m^{-2} of body surface area (BSA) commencing at time zero (BSA = N [height (m) × weight (kg)]/3600).[20] Heparin was given with the Intralipid infusion; an intravenous bolus of 200 units was given at the start of the Intralipid infusion, followed by a constant infusion of 840 units h^{-1} m^{-2}. After 20 min, the hyperglycaemic clamp was started by giving an intravenous priming bolus of 50 % dextrose (5.5 g m^{-2}) over 30 s. From 30 min onwards the plasma glucose level was maintained at approximately 9 mmol l^{-1} by a variable rate infusion of 50 % dextrose adjusted every 10 min up to 120 min. At 110 min, a 5 g intravenous bolus of arginine was given, while the dextrose, Intralipid/heparin or saline infusions were continued.

Blood samples were collected every 10 to 20 min for the estimation of plasma glucose, serum NEFA, insulin, C-peptide, TG, 3-hydroxybutyrate (OHB), and glycerol levels. After the bolus injections of glucose and arginine, samples for plasma glucose and insulin were collected every min for 4 min and then every second min up to 10 min. Samples were chilled, centrifuged at 4 °C and the supernatant stored at −20 °C until analysis. NEFA levels were measured within 2 days of collection. Some degree of *in vitro* lipolysis may have occurred after collection of the specimens; consequently our reported NEFA and glycerol levels are artefactually high. The NEFA levels we obtained during the Intralipid/heparin infusion (approximately 3000–4000 μmol l^{-1}) are in keeping with a previous study employing a similar protocol,[21] higher than those obtained in another study that used a lipoprotein lipase inhibitor in the collecting tubes (Paroxon, Sigma);[22] this was not used in our study because of concern about its toxicity to laboratory staff.

Plasma glucose was measured by a glucose oxidase method (YSI 2300 STAT plus analyser, Yellow Springs Instruments Co. Inc., Ohio, USA). Serum insulin and C-peptide levels were determined by in-house radioimmunoassays (RIA). The insulin assay method was a modification of Morgan and Lazarow,[23] with an intra-assay variation of 6% and inter-assay variation of 7 % at 30 pmol l^{-1}. The C-peptide assay was modified from Kuzuya *et al.*[24], CV 4 % and 6 % at 0.333 nmol l^{-1} respectively. The serum NEFA levels were determined by using an acylCoA oxidase based colorimetric kit (WAKO NEFA-C, WAKO Pure Chemical Industries, Osaka, Japan). Colorimetric enzymatic assays (Sigma diagnostics, Sydney, Australia) were also used to determine serum TG, glycerol and OHB levels.

Calculations and Statistical Analyses

To determine whether NEFA elevation affected insulin elimination in this study,[25] the per cent hepatic insulin extraction was estimated according to the method of Binder *et al.*[26] with % extraction = 100 × [incremental C-peptide area − incremental insulin area]/[incremental C-peptide area] and calculated on a molar basis for both C-peptide and insulin.

The serum insulin responses were analysed according to five time periods. The first, basal, −15 to 0 min; the second, 0–20 min period, during which only Intralipid or saline was infused, assessed the acute effect of elevated NEFA levels on basal insulin secretion; the third, following the bolus injection of glucose, 20–30 min, assessed acute glucose-stimulated insulin secretion; the fourth, during the hyperglycaemic clamp, 30–110 min, assessed second phase insulin secretion; and the fifth, following the arginine bolus, 110–120 min, assessed non-glucose-stimulated insulin secretion (during matched hyperglycaemia). The average rate of glucose infusion over the 30–110 min period which maintained the hyperglycaemic clamp was used to indicate the whole body insulin response.

Data are expressed as mean ± SEM. Mean values were calculated on the 30 to 110 min period for serum NEFA, TG, glycerol, and OHB levels. The area under the curve was calculated for plasma glucose, and serum insulin and C-peptide levels through the various time periods. Statistical comparisons between saline and Intralipid studies were made using Student's *t*-test for paired values in the Statview SE + Graphics program (Abacus Concepts, Berkeley, CA, USA). Here the control and Type 2 DM-risk groups were examined separately. To determine if the control and Type 2 DM risk groups responded in the same or different manner to NEFA elevation, data were analysed using repeated-measures analysis of variance with one between factor (health status: control vs Type 2 DM risk) and one within factor (infusion Type: saline vs Intralipid/heparin) by the software superANOVA (Abacus Concepts, Berkeley, CA, USA).

Table 1. Subject characteristics

	Control group	Type 2 DM-risk group
Age (yr)	25.4 ± 1.5	45.8 ± 5.0[a]
Sex (F/M)	4/2	6/0
Weight (kg)	67.1 ± 4.8	70.6 ± 4.8
BMI (kg m^{-2})	21.5 ± 1.0	25.5 ± 1.3[b]
Fasting plasma glucose (mmol l^{-1})	4.8 ± 0.1	5.3 ± 0.3
Fasting serum insulin (pmol l^{-1})	25 ± 5	65 ± 30
Fasting NEFA (μmol l^{-1})	710 ± 60	720 ± 50
Fasting TG (mmol l^{-1})	0.86 ± 0.29	1.16 ± 0.21

All results expressed as mean ± SEM. [a] $p < 0.01$ and [b] $p < 0.05$.

Results

NEFA levels were suppressed below fasting levels by hyperglycaemia during the normal saline infusion, while they rose five- to six-fold above fasting levels during the Intralipid infusion (Tables 1 and 2).

The areas under the curves for plasma glucose levels were well matched between studies during the hyperglycaemic clamp and after the arginine bolus (Figures 1 and 2) for both groups. At 110 min, the time of arginine injection, plasma glucose levels were also well matched (8.3 ± 0.3 vs 8.4 ± 0.3 mmol l^{-1} and 8.5 ± 0.2 vs 8.5 ± 0.2 mmol l^{-1}; for saline vs Intralipid studies, for the control and Type 2 DM-risk groups, respectively).

The areas under the curves for serum insulin levels (Figures 1 and 2) were not significantly different between saline and Intralipid studies when normal saline or Intralipid (plus heparin) was infused alone (0–20 min period) or during first phase insulin secretion (20–30 min period). However, during the hyperglycaemic clamp (30–110 min time period), the areas under the curves for insulin levels were significantly higher during NEFA elevation than during the saline infusion. After the arginine bolus, 110–120 min, the area under the curve for insulin levels was significantly higher during the Intralipid studies in the Type 2 DM-risk group, but not in the control group ($p = 0.09$).

To confirm that alterations in insulin levels were reflecting changes in insulin secretion, we calculated hepatic insulin extraction. Areas under the curves for serum C-peptide levels, in the 0–20 min period were not significantly different between saline and Intralipid studies, however, in the 30–110 min period, C-peptide levels were higher during the NEFA elevation (Figures 1 and 2). Hepatic insulin extraction was equal in saline and Intralipid groups (Table 2). There were no significant differences in average glucose infusion rates between studies or groups (Table 2) or at individual time points (data not shown).

Serum TG, glycerol, and OHB levels followed a similar pattern to plasma NEFA and, compared to saline infusion, were all higher during the Intralipid infusion. The OHB levels in the Type 2 DM-risk group showed more variability (Table 2).

For plasma glucose, serum insulin, and C-peptide, clamp NEFA, TG, glycerol and 3-hydroxybutyrate, as well as % insulin extraction and average glucose infusion rate, the control and Type 2 DM-risk groups did not respond differently.

Discussion

This study assessed the effects of NEFA elevation on glucose- and arginine-stimulated insulin secretion. We

Table 2. Average serum parameters, glucose infusion rate, and insulin extraction during hyperglycaemic clamp

	Control group		Type 2 DM-risk group	
	Saline	Intralipid	Saline	Intralipid
Serum NEFA (μmol l^{-1})	400 ± 130	4060 ± 640[a]	290 ± 50	3680 ± 365[a]
Serum TG (mmol l^{-1})	0.72 ± 0.12	2.12 ± 0.32[a]	1.02 ± 0.24	3.12 ± 0.65[a]
Serum OHB (μmol l^{-1})	240 ± 30	470 ± 50[b]	285 ± 115	1990 ± 1050
Serum glycerol (mmol l^{-1})	0.05 ± 0.01	1.36 ± 0.18[a]	0.06 ± 0.01	1.67 ± 0.33[a]
GIR (μmol kg^{-1} min^{-1})	15.5 ± 1.8	15.3 ± 0.8	13.5 ± 1.2	13.7 ± 1.3
Insulin extraction (%)	87.1 ± 1.1	86.7 ± 1.2	87.8 ± 1.4	87.2 ± 1.9

All results expressed as mean ± SEM.
[a] $p < 0.01$ and [b] $p < 0.05$ compared to saline study for each group.

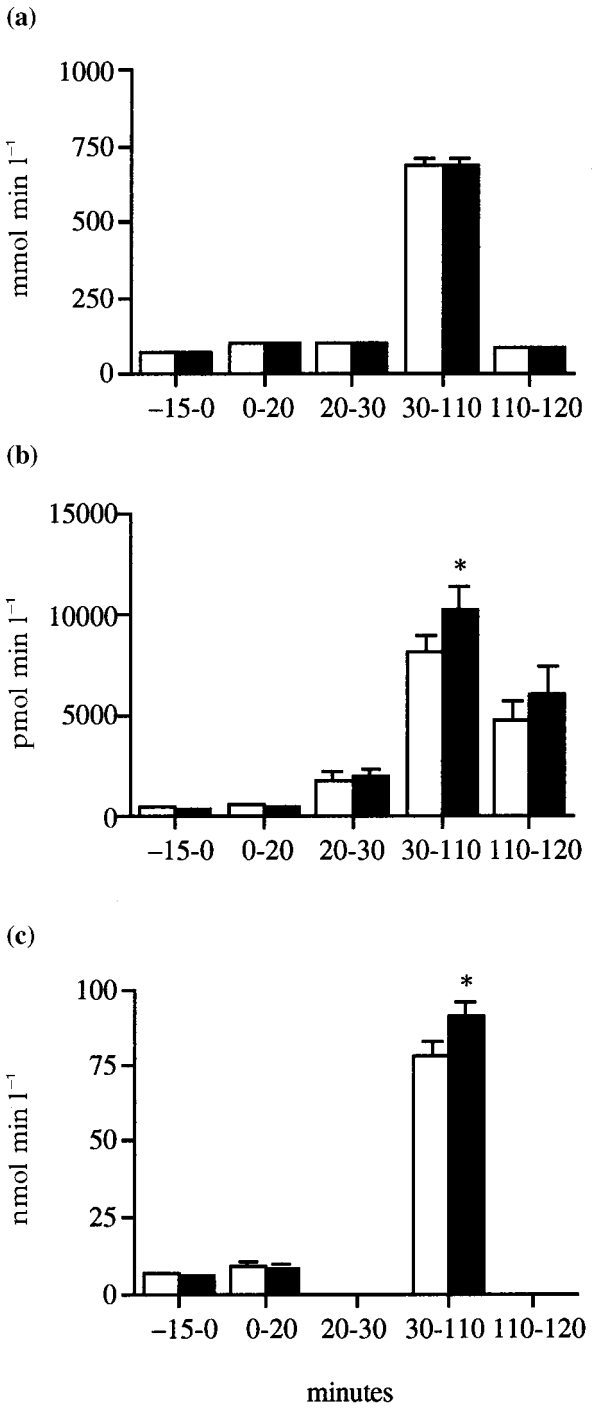

Figure 1. Area under the curve for (a) plasma glucose, (b) serum insulin, and (c) C-peptide levels in control subjects during normal saline (open boxes) and Intralipid/heparin (dark boxes) infusion studies. The time periods are the basal state (–15 to 0 min), during Intralipid or saline infusion alone (0–20 min), following the glucose bolus (20–30 min), during hyperglycaemic clamp (30–110 min) and following arginine bolus (110–120 min). C-peptide levels were not estimated for the 20–30 and 110–120 min periods. Results are expressed as mean ± SEM; *$p < 0.05$ versus saline group

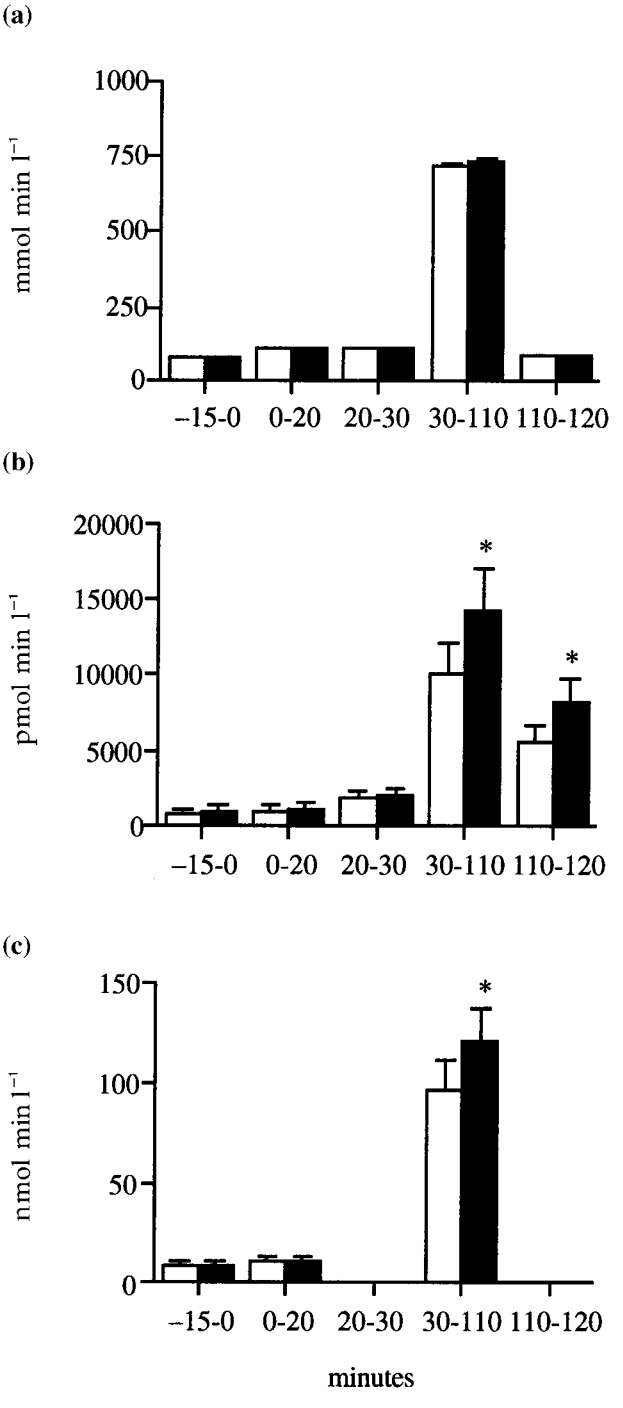

Figure 2. Area under the curve for (a) plasma glucose, (b) serum insulin, and (c) C-peptide levels in Type 2 DM-risk subjects during normal saline (open boxes) and Intralipid/heparin (dark boxes) infusion studies. The time periods are the basal state (–15 to 0 min), during Intralipid or saline infusion alone (0–20 min), following the glucose bolus (20–30 min), during hyperglycaemic clamp (30–110 min) and following arginine bolus (110–120 min). C-peptide levels were not estimated for the 20–30 and 110–120 min periods. Results are expressed as mean ± SEM; *$p < 0.05$ versus saline group

found that NEFA elevation, in normal subjects and in those with an increased risk of Type 2 DM, enhanced insulin levels during a hyperglycaemic clamp and after the additional stimulus of arginine in the Type 2 DM-risk group. As we found no significant effect of NEFA elevation on estimated insulin clearance, we interpret this as an enhancement of second-phase glucose-stimulated insulin secretion and arginine-induced insulin secretion. The variation of fasting insulin levels in the Type 2 DM-risk group suggests that this group was probably heterogeneous as regards insulin sensitivity, and they were older. However, overall the responses in this group were similar to the control group with and without elevation of NEFA levels.

No effect of NEFA elvation for 20 min was observed on insulin levels, and elevated NEFA did not affect insulin levels over the first 10 min following the glucose prime, in either the control or Type 2 DM-risk groups. We interpret this as no enhancement of first-phase glucose-stimulated insulin secretion attributable to NEFA elevation. This suggests that either first-phase glucose-stimulated insulin secretion is not affected by NEFA elevation or that a longer time of exposure to elevated NEFA levels is required before enhanced insulin secretion occurs. Other studies employing the perfused rat pancreas have shown that longer exposure (i.e. 30 min to 3 h) to elevated NEFA levels enhances acute glucose-stimulated insulin secretion.[27–29] In humans, when NEFA elevation is prolonged for 6 h, Paolisso *et al*.[15] found increased acute glucose-stimulated insulin secretion.

One other human study has examined the effects of combined hyperglycaemia and NEFA elevation on insulin secretion.[16] A similar pattern was observed to our study; however, significantly augmented serum insulin levels attributable to NEFA elevation were not found until the second day of infusion even though serum C-peptide levels were augmented during the first day as well. Also in general agreement with our study, an infusion of Intralipid plus heparin to nicotinic acid-treated rats has produced a supranormal glucose-stimulated insulin secretion during a hyperglycaemic clamp.[30] In a follow-up study in rats, pancreases perfused with long-chain saturated fatty acids were shown to be more potent at enhancing glucose-stimulated insulin secretion than medium chain or unsaturated fatty acids.[31] Intralipid is largely made from soybean oil in which the triglycerides contain 14.5 % saturated fatty acids, and 23.2 % and 56.5 % monounsaturated and polyunsaturated fatty acids respectively.[32]

It has been documented that a glucose-fatty acid cycle interaction occurs in islet cells,[33] and fatty acid products such as malonyl-CoA and long chain acyl-CoAs have been implicated in NEFA enhanced glucose-stimulated insulin secretion, with the long chain acyl-CoA levels correlating best with insulin secretion.[34] Glucose stimulation of β-cells increases malonyl-CoA and long chain acyl-CoA levels, and Prentki and Corkey[35] have suggested that in β-cells, malonyl-CoA is the regulator of fatty acid oxidation while long chain acyl-CoAs are the effectors that enhance NEFA associated glucose-stimulated insulin secretion. Moreover, when the conversion of glucose to malonyl-CoA is blocked at the penultimate step (by inhibition of ATPase citrate lyase), glucose-induced insulin secretion is severely reduced.[36] For fatty acids to exert their insulinotropic effect, as in this study, accumulation of significant amounts of malonyl-CoA, and/or conversion to long chain acyl-CoAs may be required.

In this study, arginine in the presence of elevated NEFA levels increased insulin levels during clamped hyperglycaemia. Although the response was not large it seems that the effect of NEFA on insulin levels is not specific for glucose stimulation. Thus, the elevated NEFA levels often found in Type 2 DM could contribute to maintenance of an apparently normal insulin response to non-glucose secretagogues, and contribute to the response to a combined glucose-amino acid stimulus after a normal meal.

First degree relatives of people with Type 2 DM do not always have abnormalities of lipid levels[37,38] but a subtle impairment in suppression of lipolysis has been reported.[39] In addition elevated NEFA levels have been associated with the risk of development in Type 2 DM.[40] We speculated that the β-cell response to elevated NEFA levels might differ between normals and subjects at high risk for development of Type 2 DM. However, although there was a tendency towards a greater magnitude of insulin and C-peptide response to elevated NEFA levels in the Type 2 DM-risk group, this was not statistically significant. Our study was not large and a Type II error is possible. The normals and Type 2 DM-risk groups were not matched for age and BMI, and it is possible that the greater age and BMI in the Type 2 DM risk group (while still being glucose tolerant) might be expected to generate an increase in insulin response if associated with a decrease in insulin sensitivity.[41]

In this study, increases in insulin levels were paralleled by increases in C-peptide levels, and estimated hepatic insulin extraction was similar in both the saline and Intralipid studies, suggesting that the increases in insulin levels were mainly from enhanced insulin secretion rather than reduced degradation.

There was no significant difference in glucose infusion rates between the saline and Intralipid studies, suggesting that the modest increase in insulin secretion was accompanied by some impairment of insulin action so that enhanced insulin secretion and insulin resistance were in balance.

Heparin was given with Intralipid because it activates lipoprotein lipase, thereby converting serum TG into glycerol and NEFAs.[42] Glycerol which has previously been shown to increase hepatic glucose production (HGP) during insulinopaenia and hyperglucagonaemia, accounts for some of the effect of a TG-heparin infusion on HGP, but had no effect on peripheral insulin resistance under those conditions.[4] Thus elevated glycerol levels

in this study are unlikely to have caused the impaired insulin response. Glycerol and TG at similar levels to those reached in this study have been previously shown not to increase insulin secretion significantly in humans.[11] Although ketone bodies have been shown to increase insulin secretion *in vitro*, they are weak secretagogues at the levels obtained in this study.[43] So the effects on insulin secretion in our study are largely attributable to NEFAs.

This study demonstrates that short-term elevation of NEFA levels modestly enhances the insulin response to mild hyperglycaemia and to arginine, and is consistent with NEFAs having a role in modulating glucose- and amino acid-induced insulin secretion. It provides support for the concept that hyperinsulinaemia and insulin resistance are induced in a parallel fashion in the presence of elevated NEFA levels[1] and thus may contribute in the evolution of Type 2 DM.

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